

P.5650

A NOTE ON THE AMMONIUM SULPHATE PRE-
CIPITATION OF THE ACTIVE PRINCIPLE OF
THE CULTURE FILTRATES OF *C. DIPHTHERIAE*

BY

ARTHUR FREDERICK WATSON

AND

ELSIE LANGSTAFF

201

[FROM THE BIOCHEMICAL JOURNAL, Vol. XXI, No. 2, 1927]



CAMBRIDGE
AT THE UNIVERSITY PRESS
PRINTED IN GREAT BRITAIN

LXII. A NOTE ON THE AMMONIUM SULPHATE PRECIPITATION OF THE ACTIVE PRINCIPLE OF THE CULTURE FILTRATES OF *C. DIPHTHERIAE*.

By ARTHUR FREDERICK WATSON AND ELSIE LANGSTAFF.

From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

(Received March 1st, 1927.)

IN previous papers [Watson and Wallace, 1924, 1, 2; Watson and Langstaff, 1926] the methods of preparation and some properties of the acid-precipitable specific principle of culture filtrates of the diphtheria bacillus have been described. The investigation has been extended to the examination of the precipitates produced by various concentrations of ammonium sulphate, the activity of the precipitates being again estimated by the *in vitro* flocculation test of Ramon [1922]. Throughout the paper the term "active principle" has been used to denote the substance (or substances) present in culture filtrates of *C. diphtheriae* which gives this specific *in vitro* flocculation with antitoxin. While it is probable that these flocculation values are by themselves rarely a reliable index of the antigenic value of the solutions, they obviously afford a valuable method of estimating the combining power of solutions obtained by chemical methods of purification of culture filtrates.

Heineman [1908] utilised the ammonium sulphate method for concentrating diphtheria toxin, and in one case obtained a solution ten times as strong as the original filtrate. Some loss of specific principle—as measured by animal tests (guinea-pig M.L.D.)—always occurred. Seibert and Long [1926], in a recent investigation of the chemical composition of the active principle of tuberculin, used glacial acetic acid and ammonium sulphate as precipitants. While the precipitation was always incomplete with the former, complete saturation with ammonium sulphate appeared to precipitate the whole of the tuberculin activity and the authors advanced experimental evidence to show that the active principle was a whole protein.

EXPERIMENTAL.

The solutions of the crude active material were prepared, except where otherwise stated, by inoculating media made by the three-day tryptic digestion of horse muscle [Watson and Langstaff, 1927] with p.w. 8 strain of *C. diphtheriae* and growing for ten days in the incubator. The cultures were then treated with 0.5 % toluene, 0.5 % phenol or 0.75 % of neutralised formalin

(40 % formaldehyde), filtered after 24 hours and tested for Lf value¹. The culture filtrates were grouped according to Lf values.

For the removal of ammonium sulphate or acid-precipitable material small volumes were filtered through paper. Where large volumes were being dealt with the precipitates were recovered quantitatively by centrifugation in a Sharples' centrifuge, the bowl of which was provided with a detachable celluloid roll, although it is of interest to note that Siebert and Long found the high specific gravity of their ammonium sulphate-tuberculin mixtures (containing glycerol) precluded this. The acid-precipitable material was dissolved in caustic soda to p_H 8.0 and the sulphate-precipitable material in distilled water, in each case to the original volume or to an aliquot part. The very small amount of ammonium sulphate present in these purified solutions seemed to interfere in no way with the flocculation test with antitoxin, although it was difficult to test solutions containing more than traces of the salt owing to rapid precipitation of the serum proteins. One of the features of the testing of the more concentrated solutions was the extreme rapidity of the flocculations. Very active concentrated solutions frequently flocculate in a few minutes at 37°. On diluting these solutions the time of flocculation gradually increases, and this time is not materially altered whether the diluent is water, tryptic digest broth, muscle extract or a two per cent. solution of peptone. The organic nitrogen of the purified solutions containing small amounts of ammonium sulphate was estimated after removing the ammonia with potassium bicarbonate [see Haslam, 1905].

1. *The precipitation of the active principle at various levels of ammonium sulphate concentration.*

To equal volumes of culture filtrates which had been treated with formalin, readjusted to p_H 8.0, filtered through a Berkefeld candle and incubated for a week at 36°, varying amounts of ammonium sulphate were added and the precipitates produced at the complete, two-thirds, one-half and one-third saturation levels were examined. The mixtures were allowed to stand overnight before filtration. The precipitates were washed with ammonium sulphate solution at the corresponding level and then dissolved in water. Estimations of the organic nitrogen and the specific activity of the solutions were made.

The filtrates from these initial precipitations were brought up to complete saturation by addition of solid ammonium sulphate and the second precipitates were washed, collected, dissolved in water, and tested as before.

It will be seen from Table I that the maximum recovery of the active principle is effected by complete saturation with ammonium sulphate, although similar recoveries can be made by precipitation in two successive

¹ As used in this paper the Lf dose may be defined as that volume of any solution containing the active principle of the culture filtrates of *C. diphtheriae* which is equal to one unit of antitoxin by the flocculation test (for fuller definition and description of the test see Glenny and Okell [1924] and Glenny and Wallace [1925]).

stages as shown. For the toxoid under consideration, however, the most pure active precipitate was obtained by two-thirds saturation—the precipitate containing 162 Lf units per mg. of nitrogen. The original toxoid contained 4 Lf units per mg. of nitrogen, so that a relative nitrogen purification of over 40 was effected.

Table I. *Precipitation of a diphtheria toxoid at various levels of saturation with ammonium sulphate.*

Original toxoid. TME 146 containing 14,000 Lf units per l. and 4 Lf units per mg. N.

Degree of saturation	Initial recovery of Lf units				Recovery of Lf units by completing saturation of filtrates from first precipitate			
	Lf units pptd. from 1 l. toxoid	Recovery %	Organic N pptd. from 1 l. (mg.)	Lf units per mg. N of ppt.	Lf units pptd. from 1 l. toxoid	Recovery %	Organic N pptd. from 1 l. (mg.)	Lf units per mg. N of ppt.
Complete	11,700	84	248	47	—	—	—	—
$\frac{2}{3}$	11,500	82	71	162	200	1	102	2
$\frac{1}{2}$	2,600	19	62	42	9,600	69	172	56
$\frac{1}{3}$	0	0	14	0	11,700	84	382	31

2. *The activity of the precipitates produced from one toxoid, (a) by varying the hydrogen ion concentration with acetic acid, (b) by saturating with ammonium sulphate.*

Experiments were next carried out to compare the efficiency of acetic acid and ammonium sulphate as precipitants. A typical experiment may be described. A volume of diphtheria toxoid was prepared and the acetic acid precipitation zone was determined, as described in a previous paper [Watson and Langstaff, 1926]. The p_H limits of this zone were found to be 3.1 to 4.35. Four litres of the toxoid were then precipitated at each of eight points within this zone and the precipitates recovered quantitatively by means of a Sharples' centrifuge. The total solids, total nitrogen content and the specific activity of the precipitate dissolved in alkali to p_H 8.0 were then estimated. A similar volume of the same toxoid (p_H 7.9) was completely saturated with ammonium sulphate and the activity of the precipitate dissolved in water estimated in the same way. The results are given in Fig. 1.

By varying the hydrogen ion concentration of the toxoid, maximum precipitation was produced at p_H 3.9, the isoelectric point of the active "protein" (Curve A). Correlated with this, the precipitate at this point was most active (Curve B). Curve C shows the concentration of Lf units per 20 mg. nitrogen of the precipitates produced at various points in the p_H zone 3.1–4.35. The maximum activity was 4100 units per 20 mg. nitrogen, and since the original toxoid contained 37 units per 20 mg. nitrogen a relative purification of 110 was effected by precipitation at the isoelectric point. Even at this point, however, only 39 % of the total active principle was recovered.

On the other hand, by completely saturating the toxoid (p_H 7.9) with ammonium sulphate, the whole of the activity was recovered in the precipitate (Curve D). As Seibert and Long [1926] have found with tuberculin, ammonium

sulphate is a far more energetic precipitant than acetic acid even at the isoelectric point. In fact a very strong resemblance (apart from heat stability) between the behaviour of the active principle of tuberculin and a true bacterial toxin such as diphtheria toxin may eventually be proved to exist.

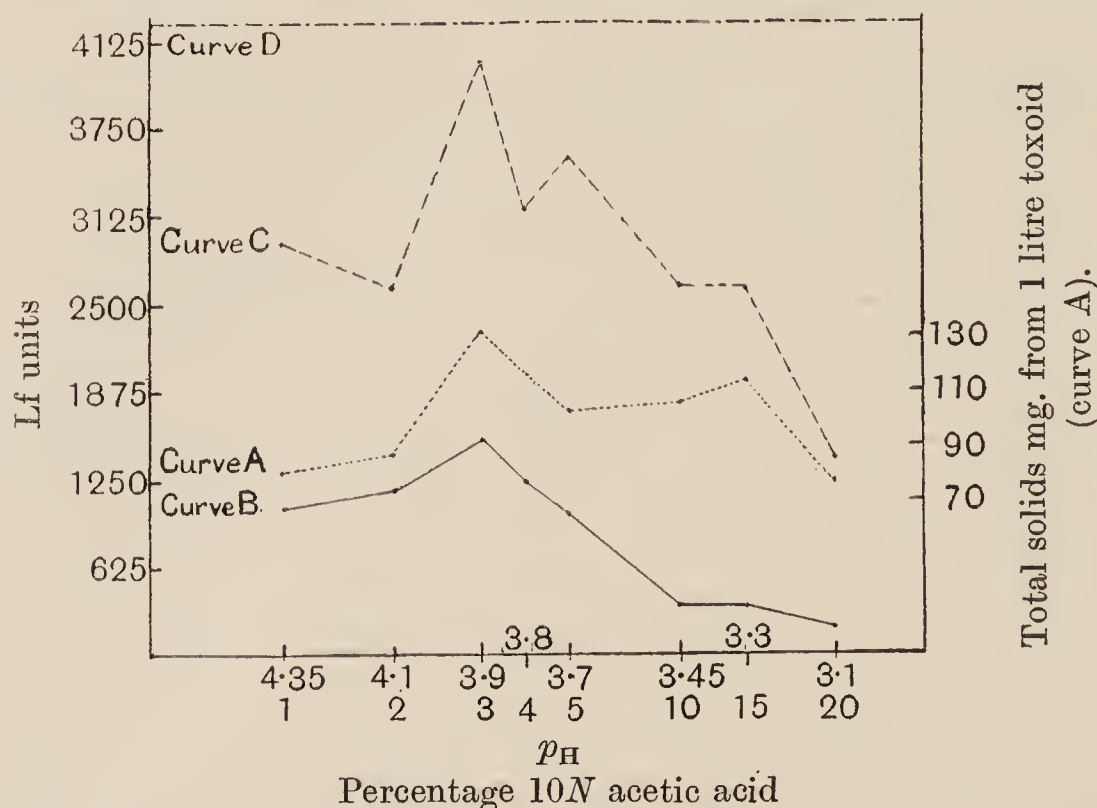


Fig. 1. The precipitability of a diphtheria toxoid by change of p_H with acetic acid and by ammonium sulphate.

Curve A. Total solids precipitated from 1 l. of toxoid at various c_H .

Curve B. Specific activity (Lf units) of various precipitates.

Curve C. Lf units per 20 mg. N of the precipitates.

Curve D. Specific activity (Lf units) of the precipitate produced by complete saturation of the toxoid (p_H 7.9) with ammonium sulphate.

3. *The influence of the preservative used for culture filtrates on the precipitability of the active principle.*

The particular chemical agent used for the destruction of the bacilli at the end of growth and the preservation of the culture filtrates during subsequent manipulation may exert a definite influence on the recovery of the active principle by precipitation with ammonium sulphate. The chief agents employed for this purpose are phenol, formaldehyde and toluene, but of these, toluene is the only one that apparently has no action on the active principle. Table II shows the recoveries from a series of culture filtrates treated with different preservatives.

In all cases except two (TB 182 and TX 179—beef-heart peptone media) the media were prepared by the three-day tryptic digestion of horse muscle [Watson and Langstaff, 1927]. In the case of the filtrates treated with phenol, very little of the active principle is found in the precipitate, although examination of the amounts of nitrogen in the material precipitated in the case of unpreserved, toluene- or phenol-treated culture filtrates gave the same results. These low recoveries from phenol-treated filtrates may be due to the "salting out" of the antiseptic and the high concentration localised in the precipitate effecting severe destruction of the active principle [see Pope, 1927]. The

recoveries from unpreserved or toluene-treated filtrates are uniformly high and in some cases practically complete. On the other hand, the formalin-treated filtrates fall into two groups, one giving practically complete recovery and the other comparatively small. In every case where the yields were high the parent toxoid was well matured—*i.e.* the formalin had been in contact with the culture filtrates for a prolonged period and the amount of free formalin had been reduced to a minimum. Further, the p_H of the toxoid had been readjusted to 8.0. The low recoveries were produced from culture filtrates which had been in contact with formalin for a short time only. It would appear that the contributing cause to the low recoveries in these cases is a p_H effect. This effect is examined in the next section.

Table II. *The effect of the presence of preservative on the activity of the precipitate produced by complete saturation with ammonium sulphate.*

No.	Preservative	No. of Lf units per 100 cc.	Lf units recovered by complete saturation with (NH ₄) ₂ SO ₄	Percentage yield
TME 172	Formalin	1100	1050	96
TME 93	"	425	425	100
CES 171 A	"	1150	550	48*
TME 150	"	1050	1000	95
TTE 154	Toluene	850	750	88
CES 169 A 3	"	1900	1800	95
TTE 181	"	1400	1200	86
TE 178 A	Phenol	1400	350	25
TE 177	"	1600	500	31
TB 182	"	1100	200	18
TX 179	Unpreserved	1200	1200	100
"	Toluene	1200	1200	100
"	Formalin	1200	600	50*
"	Phenol	1200	200	17

* Formalin added a few hours before saturation with ammonium sulphate.

4. *The influence of the hydrogen ion concentration of the culture filtrates on the "salting out" effects of ammonium sulphate.*

Very few data exist in the literature on the salting out of proteins (except perhaps those of serum) by ammonium sulphate. It is probable that the effects produced with any one protein are characteristic only for that protein. With the object of testing the effect of salting out the *diphtheriae* "proteins" in acid and alkaline solutions, a series of culture filtrates were adjusted to different p_H and then saturated with ammonium sulphate. The activity of the precipitates was tested in the usual way. The results are collected in Fig. 2.

It will be seen that complete recovery of the active principle is usually found if the salting out is conducted in slightly alkaline solution (p_H 8.0). At points on the other side of neutrality the recovery may not be complete. In the acid precipitation zone (p_H *ca.* 4.0) very low recoveries are always experienced. This is probably caused by the partial acid precipitation of the

active principle in a form insoluble in water¹. The whole mechanism of the salting out of proteins is an obscure one but it is of importance that the recovery by precipitation with ammonium sulphate of the active principle of diphtheria toxin in a form soluble in water is only complete in the absence of certain chemical substances (*e.g.* phenol or acid) which in some way affect the structure of the active principle.

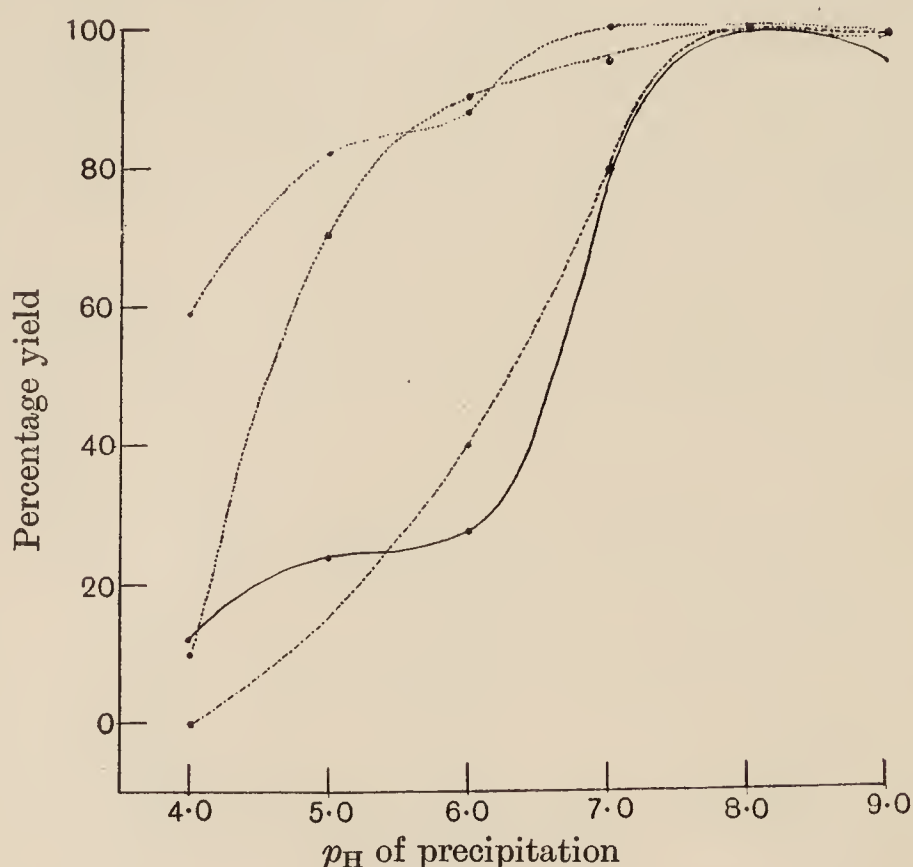


Fig. 2. The influence of p_H on the precipitation of culture filtrates by ammonium sulphate.

- - - - - • Culture filtrate untreated.
- Culture filtrate treated with 0.5 % toluene.
- Culture filtrate treated with 0.75 % formalin.

5. The chemical “purity” of the precipitates produced by acetic acid and ammonium sulphate.

In any work involving the isolation and examination of substances of physiological interest it is obviously an advantage to utilise, as a basis for attacking the problem, material as free as possible from extraneous components. To obtain some idea of the relative purity of the precipitates during the course of the investigation, the concentrations of Lf units per mg. N of the original culture filtrates and the various precipitates have been determined. Different culture filtrates vary widely in their behaviour towards the same precipitant, but the general impression received during the course of the work was that although ammonium sulphate under certain conditions may completely precipitate the active principle from the culture filtrates, the precipitates produced in the acid precipitation zone contain relatively more Lf units per mg. N. The complete examination of a low value culture filtrate is shown in Table III. Acetic acid at the isoelectric point (p_H 3.9) produced a precipitate

¹ Mastin and Schryver [1926] have recently described a well-defined acidic denatured product of albumin soluble in dilute alkalis but insoluble in dilute acids.

which was 110·8 times as “pure” as the original toxoid. The “purest” precipitate obtained in the ammonium sulphate series was that produced by two-thirds saturation where a relative purity of 28·8 was obtained (compare Table I, where the precipitate produced at this level also contained the highest concentration of Lf units per mg. N).

Table III. *The relative purity of the acid- and the ammonium sulphate-precipitable material.*

Toxoid TME 93. 1·85 Lf units per mg. N

Precipitate in zone p_H 3·1–4·35			Ammonium sulphate precipitates		
p_H of pptn.	Lf units per mg. N pptd.	Relative purification of act. principle	Degree of saturation	Lf units per mg. N pptd.	Relative purification of act. principle
4·35	150	81·1	Complete $\frac{3}{4}$ $\frac{2}{3}$ $\frac{1}{2}$ $\frac{1}{3}$		
4·1	132	71·4		17·7	9·6
3·9	205	110·8		47·2	25·5
3·8	163	88		53·2	28·8
3·7	180	97·3		27·2	14·7
3·45	132	71·3		No ppt.	—
3·3	132	71·3			
3·1	74	40			

6. *The chemical properties of the active substances.*

Apart from the presence of small traces of ammonium sulphate and of a larger amount of extraneous material, the chemical properties of the active substances obtained by salting out the culture filtrates are essentially similar to those of the acid-precipitable material previously described [Watson and Langstaff, 1926]. The salted out “proteins” can be freed from ammonium sulphate by dialysis, the loss of active principle depending largely on the nature of the membrane employed. The active material gives typical protein colour reactions and the specific substance is comparatively stable.

SUMMARY.

1. The substance which causes the specific *in vitro* flocculation with antitoxin can be salted out of culture filtrates of *C. diphtheriae* by complete saturation with ammonium sulphate. Recovery is complete in the case of filtrates untreated with any chemical agent, and adjusted to p_H 8·0 before precipitation.
2. The presence of certain chemical agents (*e.g.* phenol or acid) prevents the complete recovery of the active principle by precipitation with ammonium sulphate.
3. The material salted out by ammonium sulphate is less chemically “pure” relative to the original culture filtrate than the acid precipitate produced at the isoelectric point although, in general, the chemical properties of the precipitates are the same.

REFERENCES.

- Glenny and Okell (1924). *J. Path. Bact.* **27**, 187.
Glenny and Wallace (1925). *J. Path. Bact.* **28**, 317.
Haslam (1905). *J. Physiol.* **32**, 267.
Heineman (1908). *J. Biol. Chem.* **5**, 27.
Mastin and Schryver (1926). *Biochem. J.* **20**, 1177.
Pope (1927). *J. Path. Bact.* **31**. (In the press.)
Ramon (1922). *Compt. Rend. Soc. Biol.* **86**, 661, etc.
Seibert and Long (1926). *Amer. Rev. Tuber.* **13**, 398, 408.
Watson and Langstaff (1926). *Biochem. J.* **20**, 50.
—— — (1927). *J. Path. Bact.* **31**. (In the press.)
Watson and Wallace (1924, 1). *J. Path. Bact.* **27**, 271.
—— — (1924, 2). *J. Path. Bact.* **27**, 289.



The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

The annual subscription is 35s., which includes a copy of the Journal, and becomes due on January 1st. Further information may be obtained on application to the Hon. Sec., Dr H. D. Kay, Medical Unit, London Hospital, Whitechapel, London, E.1, or to the Hon. Treas., Mr J. Addyman Gardner, 13 Campden Grove, Kensington, W. 8, to whom subscriptions should be sent.

Papers for publication should be sent to Prof. A. Harden, F.R.S., Lister Institute, Chelsea Gardens, S.W. 1. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

The Journal is issued about every two months and the date at which each paper is received by the editors is printed at the beginning of the paper.

All communications respecting the purchase of copies of the parts or volumes whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£3 net per volume (post free) payable in advance) should be addressed to The Cambridge University Press, Fetter Lane, London, E.C. 4, or to the Hon. Treas., Mr J. Addyman Gardner, 13 Campden Grove, Kensington, W.8. For prices of back numbers and volumes see list on p. 4 of Wrapper.

Quotations can be given for Buckram binding cases and for binding Subscribers' Sets

The Cambridge University Press has appointed the University of Chicago Press agent for the sale of the *Biochemical Journal* in the United States of America and has authorised the following subscription price. \$14.25 net.

Reprinted from the Veterinary Record, May 21st, 1927.

**A Consideration of some Diseases of Animals—
The Laboratory and Field Worker.***

By Major T. DALLING, M.R.C.V.S., Wellcome Research
Laboratories, Beckenham.

The following are some of the points included in the investigation of animal diseases—a general study of the disease as it occurs naturally in the field ; a detailed study of naturally-occurring cases, preferably in the field (under certain circumstances it may be necessary to carry out this study on animals transported to a laboratory, especially if the course of the disease is prolonged); a detailed macroscopic and microscopic consideration of affected tissues, including bacteriological and similar types of examinations in an attempt to name the cause. The investigation from this point may vary considerably, depending largely on the findings. As the ultimate object of research into disease is the establishment of prophylaxis and curative treatment, it naturally follows that if definite bacteriological or similar evidence is forthcoming as to etiology, attempts will be made to evolve biological products which can ultimately be used in the field. The efficacy of such products must first be established in the laboratory on laboratory experimental animals if such are susceptible, then on small numbers of the animals naturally attacked, and finally one or more extensive field trials must take place in which an adequate number of untreated animals is exposed to the natural disease under exactly similar conditions to those treated. The issue from such an experiment would decide whether the product evolved could be adopted for general use in all parts of the country. If, during the course of the investigation, the cause of the disease is shown definitely to be non-parasitic in character, the above does not apply and a further study of preventive and curative methods must be undertaken. The following remarks apply to diseases of a parasitic nature.

The laboratory and the field worker play equally important parts in investigations. To a large extent the veterinary practitioner is the field worker, and we cannot emphasise too strongly the part he can, and often does, play, and the very great help he can be to the laboratory worker. The further we study disease problems the more we must agree that in cases where the veterinary practitioner plays his part well, the earlier can results be got and the more reliable are the results of tests carried out. There are times when the laboratory worker must become

*Presented at the Annual Meeting of the North Wales Division, Bangor, February 25th, 1927.

his own field worker, for till very recently veterinary practitioners apparently took little interest in the diseases of some animals, *e.g.*, poultry, sheep, pigs, etc., but every year sees a larger number taking up the treatment of diseases of such animals and becoming of real value in further investigations.

The early and the final stage of investigations should be mainly in the hands of the field worker. Granted that the naming of the cause and pathological changes and the preparation of products for field use must be the work of the laboratory worker entirely, the field worker's share covers practically everything else, from the diagnosing of cases, the ensuring of a constant supply of investigation material, the obtaining of facilities to carry out field experiments and trials in biological products, to the final testing of preventive and curative measures on an extensive scale.

During the last three years of investigation into diseases of animals, we have tried under practically every condition to have a veterinary practitioner to play the part of the field worker. In a few cases this was impossible, either owing to the non-existence of a practitioner in the district in which the disease under study largely existed, or to the practitioner having little interest in the condition. This refers mostly to poultry investigations, yet we look forward to the very near future when poultry diseases will be included in the daily routine of the practitioner, a position in the creating of which recent and future research will, we believe, play a big part.

The diseases we wish to consider are *Blackleg in Cattle*, *Leptospiral Jaundice in Dogs* and *Bacillary White Diarrhœa in Poultry*, because they illustrate some of the points already mentioned, and some of the work affords examples of the results of collaboration between the laboratory and the field worker. It is mostly with the etiology and treatment that we propose to deal.

BLACKLEG IN CATTLE.

From 1887, when Roux investigated blackleg in cattle, till very recently, *B. chauvoei* was generally regarded as the sole cause of the disease. Even to-day there are some who incline to this view and hold that any other organisms that may be isolated from lesions of blackleg are either extraneous contaminators or are organisms which have caused a pathological condition very similar to blackleg, but still not true blackleg. The organism, other than *B. chauvoei* most commonly associated with blackleg lesions is *Vibrion septique*, which formerly was said to cause malignant œdema. Weinberg (1) in an article published lately, produces a table illustrating the finding of thirteen groups of Continental workers who examined material from 487 cases of blackleg. In 223 cases *Vibrion septique* was isolated, sometimes alone and sometimes in

association with *B. chauvoei*. Workers in this country have not had such an experience, though *Vibrion septique* has been isolated in our laboratory quite recently from a case of blackleg; the organism was not associated with *B. chauvoei*. Bearing in mind the facts that outbreaks of blackleg, no matter what is the infecting organism, arise from wound infection with the organism which may be harboured in the soil, that the anærobic group of organisms has some members pathogenic for cattle, as seen by experimental inoculation, and that *Vibrion septique* is highly pathogenic to cattle, is more or less ubiquitous in nature and when inoculated experimentally produces lesions indistinguishable from those produced by *B. chauvoei*, it seems fairly certain that under natural conditions *Vibrion septique* will invade the animal body *via* wounds and produce clinical blackleg. The evidence in this country of *Vibrion septique* infection in clinical blackleg is not yet complete. We feel that many more outbreaks should be studied in this country to establish once and for all if organisms, other than *B. chauvoei*, may produce natural clinical blackleg. The ease with which contaminant organisms may be included in samples of muscle taken for such examinations must be borne in mind, both by the field and the laboratory worker, as well as the difficulty experienced in growing, isolating and identifying *B. chauvoei* compared with some of the other pathogenic anærobes, and the possibility therefore that it may be missed in contaminated material and a false position of importance allotted to the contaminants.

Because of the belief that *Vibrion septique* may play a not unimportant part in the causation of blackleg, some recent vaccines to protect against this organism and *B. chauvoei* have been made in this country and on the Continent. We have shown on several occasions that a *Vibrion septique* toxin-antitoxin mixture can be made, which, after two injections, will produce a high degree of immunity in guinea-pigs and in sheep; a dose of toxin or culture lethal to unprotected animals is tolerated with ease by those protected by the mixture. It has also been shown that a moderate degree of immunity results after one injection of such a mixture. Further, it has been shown by many, including ourselves, that complete immunity may be found in guinea-pigs and sheep following a single dose of *B. chauvoei* filtrate. Equal parts of *Vibrion septique* toxin-antitoxin mixture and *B. chauvoei* filtrate are used to make the vaccine referred to. Field trials of this vaccine have been satisfactory, but all field workers who have carried out the tests are not in favour of two doses being given, because of the expense incurred in the inoculations. At the present time there is no evidence to show that any preparation made by using *Vibrion septique* will, after one injection, produce a high degree of immunity in the field, though McEwen (2) has recorded results of some work on the subject which show promise

that such a product may be evolved. One dose of *B. chauvoei* filtrate, however, will completely immunise. If it is desirable to use the double vaccine and only one dose can be administered, complete protection against *B. chauvoei*, and some protection against *Vibrion septique* will result. Some protection is better than none at all; hence, even though only one dose may be injected, it would appear that the double vaccine is indicated in the light of our present day knowledge.

For the treatment of cattle affected with blackleg, doubtless every practitioner has his own pet remedy. It may be of interest to know that some practitioners rely on the injection of anti-*B. chauvoei* serum. Such a serum can be produced and some laboratories issue a serum which, when tested in the laboratory, has the power to check infection by *B. chauvoei*.

Lately, it was necessary, for some laboratory work, to use an anti-*B. chauvoei* serum and we made some in a cow by injecting *B. chauvoei* filtrate.

(Table 1 shows that the serum was a true anti-bacterial *B. chauvoei* serum.)

TABLE 1.

Blackleg Serum—Made in Cow.

Guinea-pigs received serum two days before culture.

Serum.	Culture.	Result.
5 c.c.	0.2 c.c.	L.
2.5 c.c.	0.2 c.c.	L.
1.0 c.c.	0.2 c.c.	L.
1.0 c.c.	0.1 c.c.	L.
Normal Cow Serum	0.1 c.c.	D.
No Serum	0.1 c.c.	D.
	0.1 c.c.	D.

L = Lived.

D = Died.

High grade *Vibrion septique* antitoxin can also be produced.

CANINE LEPTOSPIRAL JAUNDICE.

Okell, Dalling and Pugh (3) intimated that cases of canine leptospiral jaundice had been studied and *Leptospira icterohaemorrhagica* isolated. We believe this parasite to be the cause of outbreaks of canine jaundice or "yellows"—a disease which occurs as an enzootic in many kennels. It must be pointed out here that the name canine leptospiral jaundice or "yellows" does not include sporadic cases of jaundice which the practitioner frequently sees in dogs, but refers to that highly-fatal infectious disease during the course of which many or all of the dogs in a kennel may be affected. The disease has been reported to us from all over England and Scotland and is most commonly found affecting young dogs in sporting kennels.

The parasite is found normally in the kidneys of male wild rats, and would appear to be harboured there without interfering with the general health of the animal. It is

voided in the urine and may thus soil and contaminate food, litter, etc., to which the rats have access. Houston (4) has demonstrated the presence of leptospira in well water on many occasions, and in some cases it would appear that rats could not have had access to the water. Okell has been able, working with water samples from the same wells, to show that one sample contained leptospira which were pathogenic for tame rats and guinea-pigs. Many leptospiræ were seen in the other samples, but on experiment they proved non-virulent. Buchanan (5) working on slime obtained from parts of coal mines in Scotland was able to demonstrate living, virulent leptospiræ which were believed to have caused cases of Weil's Disease in some miners working in the mines. The slime was in such a position that rats could not have had access to it. It would appear, then, that though rats are the important vectors of the disease in this country, other sources do exist, though possibly in the case of dog infections they are of little consequence.

It is a very simple process to infect dogs with virulent parasites in the laboratory. Whereas our original method was to inject a suspension of mashed up guinea-pig liver or cultures intraperitoneally, we have shown that infection can be brought about by feeding the organism in the food and by scratching the dog's skin and laying infected material on the scratched area. In all our recent infection experiments we have used the scratch method with complete success. We can, therefore, now definitely state that dogs can be infected by picking up infected material in food or water and through skin abrasions on any part of the body, including the pads of the feet.

Treatment of leptospiral jaundice is more successful from a prophylactic than a curative aspect. Serum and vaccine have been prepared and have been shown to be effective.

Serum is made in horses by repeated injections of killed or attenuated organisms. In the laboratory it has been shown to protect guinea-pigs and dogs when administered before, at the same time as, or following the living parasites. In practice its value as a curative agent is very problematical because of the rare occasions on which it can be injected early in the course of the disease. Experiments already reported show that icteric symptoms develop about 4—5 days after infection has taken place, and that when gross infection is present, serum can do little to save the animal's life after jaundice is established. Its value is its ability to produce a passive immunity of about three weeks' duration, and therefore its chief use is in preventing the spread of the disease in an infected kennel. Inoculations done on all the dogs in a kennel in which one or more cases have been diagnosed will prevent their infection and allow time for the removal of the infecting agent. We have much proof on this point. Although we have shown experimentally that the appearance of icterus in

the very severe infections produced in laboratory work marks the end of the usefulness of the serum, many veterinary surgeons have had good results from its use, even at this stage of the disease. The results reported to us vary, but there is some chance that in the field, even in dogs showing marked icterus, recovery may occur after injection of serum.

Vaccine treatment has given good results in the laboratory and in the field. Its use should be confined to dogs in kennels in which periodic outbreaks of jaundice occur, and in which no actual cases have occurred for some time prior to its use. During the period immediately following injection the dog may be more susceptible to the action of the parasite, hence care should be taken that no cases of disease are present at the time of vaccine injection. Our former experiments have been carried out with vaccine made from guinea-pig liver; lately we have succeeded in culturing leptospira on artificial media in bulk and have experimented on dogs with a vaccine made from such cultures. Complete protection to tests of virulent material results, following two inoculations of treated culture in doses of even 0.1 c.c.

TABLE II.

Leptospira Vaccine—Made from Cultures.

Dogs 69—71	0.1 c.c.	7/8/26 and 20/8/26
„ 72—74	1.0 c.c.	„ „ „
„ 75—77	0.1 c.c.	20/8/26 and 27/8/26
„ 78—80	1.0 c.c.	„ „ „
All dogs tested 10/9/26.		
Dogs 69—80—All lived.		
„ 81—85 (which had received no vaccine)	—All died from jaundice.	

BACILLARY WHITE DIARRHŒA IN FOWLS.

We refer to this disease because of its recent increasing prevalence and because we feel that the veterinary practitioner must be in a position to advise on its prevention and cure. The rate of spread of B.W.D. all over the country must be expected from the numbers of all kinds of fowls being bred from to-day without adequate supervision by authorities on disease of poultry. The annual losses in chicks from B.W.D. are enormous and the present hatching season is even worse than the last, from reports already to hand.

B.W.D. is fatal to young chicks only, but adult fowls may harbour the infecting agent (*B. pullorum*) while appearing perfectly normal and healthy. Such fowls are termed “carriers,” and they are responsible for the perpetuation of the disease from year to year. Rettger and others have worked out the cycle of infection. We have confirmed this work in this country. Chicks may be hatched out in an infected state, or, in some cases, may be

infected during the first few days of life by picking up contaminated excreta, etc. The mortality among such chicks varies greatly, depending on the virulence of the infecting agent. Practically every chick in the hatch may be wiped out, or, if the infection is light, the death rate may be extremely low. Survivors in the infected hatch often become "carriers," *B. pullorum* being located in the ovary and infecting many of the eggs laid when the chick reaches maturity, so that the offspring of such hens in their time produce chicks which die of B.W.D., infect other chicks or become "carriers."

We have studied methods for the prevention of the disease, realising very early in our work that curative methods were unpractical. Two points have interested us specially: prevention of spread among chicks and diagnosis of the carrier fowl. Experiments now in progress indicate that where there is reason to suspect B.W.D. from hatching eggs, the dividing of the young chicks into small units tends to prevent its spread. This is in keeping with epidemiological expectations and only concerns the chicks hatched from non-infected eggs in contact immediately after hatching with infected chicks. There is little hope for the chick from infected eggs; our aim must be to prevent the breeding from fowls capable of producing infected eggs, and, if this is not practical, to prevent the spread from chick to chick.

Small lots of an anti-serum have been made by injecting hens, rabbits, sheep and goats with *B. pullorum*. Laboratory tests with this serum have shown that this disease can be prevented in healthy chicks provided the serum is injected at the same time as, or prior to, the feeding or injecting of the causal organisms. Field trials have been done on a limited scale, but in an experiment in which over 2,000 day-old chicks, bred from hens in which about 50 per cent. were shown to be "carriers," were injected, our conclusions were that serum does prevent infection from chick to chick, but does little to help the "egg-infected" chick. Further experiments are now in progress.

Diagnosis of the "carrier" hen can be made by carrying out agglutination tests or by the results from the intradermal wattle test. Both tests have been described by American workers. We have had occasion to study both methods and have concluded that both may be useful. It is necessary to repeat tests at intervals on all stock passed clean by either test, but we believe B.W.D. can be cleared entirely from any flock by adequate testing of the breeding fowls. The cockerel should be included in all tests done.

We hope to publish an article soon on results of our work on B.W.D.

We do not feel it necessary to point in detail to the relationship of the laboratory and field workers in the above work, but we do want to emphasise that much has been made possible by their close collaboration.

REFERENCES.

- (1) WEINBERG and GINSBOURG.—*Bull. de l' Inst. Pasteur*. Vol. XXIV. No. 20.
- (2) M'EWEN, A. D.—*Journ. Comp. Path. and Therap.* Vol. XXXIX. Part 4.
- (3) OKELL, DALLING and PUGH.—*Veterinary Journal*. Vol. 81. No. 1.
- (4) HOUSTON, A.—Metropolitan Water Board 20th Annual Report, 1925-26.
- (5) BUCHANAN, G.—*British Medical Journ.*, 1924. Journ. II. p. 990.

DISCUSSION.

In opening the discussion, Mr. MONTGOMERIE expressed his particular appreciation of the paper which Mr. Dalling had presented that afternoon. Mr. Dalling was ready to make the long journey from London and had read one of the most interesting and thoughtful papers to which it had ever been their pleasure to listen.

Proceeding, Mr. Montgomerie said, "We have had the normal course of an investigation outlined and the field worker has been assigned his due and proper place in the scheme. It might well be emphasised that the field worker, especially the practitioner, should come first on the scene. He has the opportunity to come in contact with conditions which require investigation and should be the person who draws the attention of the laboratory worker to the problem. Frequently the call comes from another source—a section of the agricultural community, a breed society or other such organisation.

"Occasionally the laboratory worker may appear to invade the field of his colleague, for the careful large scale test of a product, with the necessary controls, is often a time-consuming job which the practitioner cannot undertake. An example of this is to be found in the trial of vaccines and sera prepared for the prevention of lamb dysentery. Yet such products reach a more or less commercial stage when the observations of the practitioner-field-worker, however general they may be, are appreciated by the laboratory worker.

"The suggestion that, in this country as well as abroad, blackleg of cattle may frequently be caused by an organism other than *B. chauvoei* may appear rather disturbing. Vaccination against blackleg in cattle is one of the most successful preventative inoculations we possess—one which has brought our profession much credit. We have believed that these vaccines were made from material containing *B. chauvoei* or its products. There is no cross immunity between *B. chauvoei* and *Vibrio septique*, yet we know these vaccines are highly successful. Remembering that anaerobes have only been carefully isolated and studied in the post-war years we are entitled to wonder

if these vaccines are made from pure cultures. Yet we have been told that a double inoculation is necessary to protect against *Vibrion septique* and we know that some of the blackleg preventatives are used as a single inoculation.

"I think text-books tell us that the *B. chauvoei* generally gains entrance to the animal body through infection of a wound in the alimentary canal, and, if I remember correctly, some experiments were conducted which supported this idea. Can Mr. Dalling say if the feeding of *Vibrion septique* under conditions likely to cause injury to the alimentary canal will produce typical blackleg? That on subcutaneous injection this organism does give rise to a blackleg lesion is hardly proof, unless, of course, you accept external wound infection as the usual route.

"While two injections of vaccine made by most methods are necessary to give protection against *Vibrion septique*, McEwen has recently introduced a mode of preparing the vaccine which, by a single injection, gives protection. This piece of work is of much interest and significance. I look forward to reading the result of an extensive field trial of formalised vaccine prepared as McEwen suggests.

"I have not had any experience of anti-*B. chauvoei*, and I wonder if it is widely employed. Do practitioners see a sufficient number of blackleg cases alive to make it worth while stocking this serum? If this serum is effective does its action not favour the opinion that *Vibrion septique* is much less a cause of blackleg in this country than the figures of continental workers might suggest?

"In North Wales we hear of many losses among sheep characterised by sudden death and variously named (*e.g.*, strike), according to the locality, time of year, etc. There are those which appear to be true braxy, but I would be glad if Mr. Dalling could tell us which organism causes those deaths in Spring amongst ewes and forward lambs which we meet, or hear about.

"In offering canine leptospiral jaundice for your consideration we are presented with a splendid example of the result of good team work. One can readily picture Pugh bringing this disease, together with a wealth of clinical *data* and material, to the notice of the laboratory workers. The investigation of 'yellows' was so admirably and skilfully conducted by these three workers that it must be regarded as one of the premier achievements of recent years.

"So far, bacillary white diarrhoea has attracted little attention within North Wales, for it is only recently that poultry keeping has extended beyond the 'barn-door' type. Mr. Dalling has said that the disease is fatal to young chicks only. I would ask if he has not met the condition as an acute infection of adult birds. Is there any spread of the disease from adult birds directly to other stock, and what is the significance of the test on the

cockerel? May he transmit the disease? I am interested to hear that the intradermic method of testing may be developed, for I believe that many practitioners will prefer the latter. In using it they can read the results themselves and, without delay, yet with great certainty, they can pick out the reactors from the breeding stock.

"I am sure that each of us will wish to discuss this paper and add our quota of thanks to Mr. Dalling."

Others present having contributed to the discussion, Mr. DALLING, in reply, thanked those present for the welcome he had been given and said that the lively discussion that had taken place had amply repaid him for any trouble to which he had gone in preparing the paper and in coming to Bangor to address the Division.

The question of the cause and prevention of blackleg in cattle had been dealt with by Mr. Montgomerie and most of the other speakers. He quite agreed that the disease as it occurred in Britain would appear to be caused mainly by *B. chauvoei*. The fact that histological products made from *B. chauvoei* afforded such protection in the field would amply justify one in making such a deduction. There were, however, instances in which vaccination with such products had apparently yielded poor results. One was cited that day by Mr. Roberts, and occasionally in blackleg districts there were farms on which the disease was not fully protected against by *B. chauvoei* biological products. It would be of considerable scientific interest to investigate the cause of the so-called blackleg on such farms. Another point of which sight must not be lost was the position even to-day in the isolation and purity of anærobes, and therefore the question of the true character of agents used in the immunisation against blackleg. One inoculation of products made from *Vibrion septique* resulted in a certain number of injected animals being immunised. As far as was known, the feeding of cattle and sheep with *B. chauvoei* and *Vibrion septique* cultures had not produced any symptoms of recognisable disease. Research had still to show the necessary conditions of the various body systems for infection to take place in most diseases. From the descriptions given by Mr. Montgomerie, it was probable that some anærobic infection was prevalent in the lambs dying of so-called 'strike.' It had still to be shown that *B. chauvoei* infections occurred in sheep in this country under natural conditions. Protection experiments could be devised to show if an anærobic infection was present in the lambs, and, if so, which was the infecting agent. Investigations were at present in progress on diseases affecting ewes before and after lambing in various parts of the country.

Leptospiral jaundice was a disease quite apart from distemper, though without doubt it would attack dogs suffering from or recovering from distemper. It was caused by a *Leptospira*, while recent work had confirmed

the view that distemper was caused by an ultravisible virus.

It was evident from the discussion that B.W.D. did not exist to a great extent in North Wales. Poultry keepers there must be in a much happier position than those in the South of England. Infections did occur in adult stock and the causal agent was presumably the same as that infecting chicks. The incidence of the disease in adults was small compared with chicks, though the literature would show that "carrier" hens might, when penned up with clean birds, cause them to become reactors; recent experiments had not confirmed such work. Cockerels should always be included in tests for "carriers," as a small percentage had been shown to react.

